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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Receptor Tyrosine Kinase

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ABSTRACT OF THE DISCLOSURE

Novel receptor tyrosine kinase protein and isoforms thereof which are expressed only in cells of the endothelial lineage, and DNA segments encoding the novel protein and isoforms thereof are disclosed. Methods for identifying ligands which are capable of binding to the receptor protein and methods for screening for agonist or antagonist substances of the interaction of the protein and a ligand are also disclosed.

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Title: Novel Receptor Tyrosine Kinase

FIELD OF THE INVENTION

The invention relates to a novel tyrosine kinase receptor protein and isoforms thereof, DNA segments encoding the novel protein and isoforms thereof, and uses of the protein and DNA segments.

BACKGROUND OF THE INVENTION

Transmembrane receptor tyrosine kinases (RTKs) comprise a large and evolutionarily conserved family of structurally related proteins capable of transducing extracellular signals to the cytoplasm. The latent oncogenic potential of these molecules and the molecular mechanisms by which they function in signalling pathways have been the subject of extensive study.

In addition, genetic and biochemical analyses of a variety of developmental mutants have led to recognition of the pivotal roles played by RTK-mediated signalling pathways in the regulation of cell determination, migration, and proliferation. Notable examples in *Drosophila* include the role of *sevenless* and its ligand, *bride of sevenless*, in R7 photoreceptor determination (Krämer, H., Cagan, R.L. & Zipursky, S.L. (1991), *Nature*, 352, 207-212), and of DER/*flb* in early morphogenetic events during gastrulation (Schejter, E.D. & Shilo, B.-Z. (1989), *Cell*, 56, 1093-1104). Similarly, in the mouse, loss of function mutations at the W/c-*kit* (Geissler, E.N., Rayn, M.A. & Housman, D.E. (1988), *Cell*, 55, 185-192; Chabot, B., Stephenson, D.A., Chapman, V.M., Besmer, P. & Bernstein, A. (1988), *Nature*, 335, 88-89) and *Sl* (Russell, E.S. (1979), *Adv.Genet.*, 20, 357-459) loci have revealed the importance of the Kit receptor and its ligand in melanogenesis, hematopoiesis, and gametogenesis (Dubreuil, P., Rottapel, R., Reith, A.D., Forrester, L. & Bernstein, A. (1990), *Ann. N.Y. Acad. Sci.*, 599, 58-65;

Williams, D.E., Eisenman, J., Baird, A., Rauch, C., Ness, K.V., March, C.J., Park, L.S., Martin, U., Mochizuki, D.Y., Boswell, H.S., Burgess, G.S., Cosman, D. & Lyman, S.D. (1990), *Cell*, 63, 167-174; Copeland, N.G., Gilbert, D.J., Cho, B.C., Donovan, P.J., Jenkins, N.A., Cosman, D. Anderson, D., Lyman, S.D. & Williams, D.E. (1990), *Cell*, 63, 175-183 and Flanagan, J.G. & Leder, P. (1990), *Cell*, 63, 185-194) while a deletion in the gene encoding PDGFR-α has been correlated with the *Patch* mutation, which also causes a defect in melanogenesis (Stephenson, D.A., Mercola, M., Anderson, E., Wang, C., Stiles, C.D., Bowen-Pope, D.F. & Chapman, V.M. (1991), *Proc.Natl.Acad.Sci.*, 88, 6-10). These observations, together with others (reviewed in Pawson, T. & Bernstein, A. (1991), *Trends Gen.*, 6, 350-356), have established the importance of receptor-ligand interactions in the regulation of development.

Angiogenesis in both the embryo and adult requires the differentiation, proliferation, and migration of endothelial cells. Tissue transplantation studies with quail/chick chimeras have established that the developmental cues for both endothelial cell differentiation and proper patterning of vessels are extracellular and not pre-programmed within the cell (Noden, D.M. (1988) Development, 103, 121-140) Several peptide hormones, such as bFGF, VEGP and PD-EGF, have been shown to have both mitogenic and chemotactic effects on cultured endothelial cells (see Tomasi, V., Manica, F. & Spisni, E. (1990), *BioFactors*, 2, 213-217; Klagsbrun, M. & D'Amore, P. (1991), *Annu.Rev.Physiol.*, 53, 217-239, for reviews). However, many of these factors also show similar effects on other cell types, implying that receptors for these factors are also expressed by such cells.

Studies have demonstrated that both tyrosine kinase

activity and phosphotyrosine-containing proteins are increased in embryonic chicken heart relative to the adult (Maher, P.A. (1991). *J.Cell Biol.*, 112, 955-963), and that inhibitors of kinase activity impede inductive processes during *in vitro* differentiation of cardiac explants derived from chicken embryos (Runyan, R.B., Potts, J.D., Sharma, R.V., Loeber, C.P., Chiang, J.J. & Bhalla, R.C. (1990), *Cell Reg.*, 1, 301-313).

SUMMARY OF THE INVENTION

The present inventors have identified and characterized a receptor tyrosine kinase that plays a critical role in murine cardiogenesis. The heart forms early in mouse embryogenesis and its development is known to be accompanied by the differentiation from mesoderm of myocytes and endothelial cells that subsequently form the myocardium and endocardium, respectively (Manasek, F.J. (1976), in The Cell Surface in Animal Embryogenesis and Development, p.545-598, Elsevier/North-Holland Biomedical Press; Kaufman, M.H. & Navaratnam, V. (1981), *J.Anat.*, 133, 235-246). There have not hitherto been any reports of directed screens for tyrosine kinases expressed during murine cardiogenesis.

In particular, the present inventors using reverse transcription coupled to the polymerase chain reaction (RT-PCR) isolated from murine embryonic heart a cDNA, designated *tek*, whose deduced amino acid sequence corresponds to a novel RTK. The *tek* locus of mouse was mapped to chromosome 4. The present inventors have also shown by *in situ* hybridization that *tek* is expressed in the endocardium as well as the endothelial lining of the vasculature. *tek* was also found to be expressed in both mature endothelial cells and their progenitors, suggesting that the signalling pathways regulated by *tek* may be important to both the determination and proliferation of cells of the endothelial lineage.

The present invention therefore provides a purified and isolated DNA segment having a sequence which codes for a receptor tyrosine kinase protein which is expressed only in cells of endothelial lineage, or an oligonucleotide fragment of the DNA segment which is unique to the receptor tyrosine kinase protein of the invention. In a preferred embodiment of the invention, the purified and isolated DNA segment has the sequence as shown in Figure 1.

The invention also contemplates a double stranded nucleotide sequence comprising a DNA segment of the invention or an oligonucleotide fragment thereof hydrogen bonded to a complementary nucleotide base sequence.

The invention further contemplates a recombinant molecule comprising a DNA segment of the invention or an oligonucleotide fragment thereof and an expression control sequence operatively linked to the DNA segment or oligonucleotide fragment. A transformant host cell including a recombinant molecule of the invention is also provided.

Still further, this invention provides plasmids which comprise the DNA segment of the invention.

The invention further provides a method of preparing a novel receptor tyrosine kinase protein or isoforms thereof utilizing the purified and isolated DNA segments of the invention. The method comprises culturing a transformant host cell including a recombinant molecule comprising a DNA segment of the invention and an expression control sequence operatively linked to the DNA segment, in a suitable medium until the protein is formed and thereafter isolating the protein.

The invention further broadly contemplates a substantially

pure receptor tyrosine kinase protein or a part thereof, which is expressed only in cells of endothelial lineage.

The invention also permits the construction of nucleotide probes which are unique to the novel receptor tyrosine kinase protein of the invention or a part of the protein. Thus, the invention also relates to a probe comprising a nucleotide sequence coding for a protein, which displays the properties of the novel receptor tyrosine kinase of the invention or a peptide unique to the protein. The probe may be labelled, for example, with a radioactive substance and it may be used to select from a mixture of nucleotide sequences a nucleotide sequence coding for a protein which displays the properties of the novel receptor tyrosine kinase protein of the invention.

The invention provides a method for identifying ligands which are capable of binding to the novel receptor tyrosine kinase protein of the invention, isoforms thereof, or part of the protein, comprising reacting the novel receptor kinase protein of the invention, isoforms thereof, or part of the protein, with at least one ligand which potentially is capable of binding to the protein, isoform or part of the protein, under conditions which permit the formation of ligand-receptor protein complexes, and assaying for ligand-receptor protein complexes, for free ligand or for non-complexed proteins. In a preferred embodiment of the method, ligands are identified which are capable of binding to and activating the novel receptor tyrosine kinase protein of the invention, isoforms thereof, or part of the protein. The ligands which bind to and activate the novel receptor tyrosine kinase receptor of the invention are identified by assaying for protein tyrosine kinase activity i.e by assaying for phosphotyrosine.

In addition, the invention provides a method of using the

novel proteins of the invention for assaying a medium for the presence of a substance that affects a tek effector system. In accordance with one embodiment, a method is provided which comprises providing a known concentration of a receptor tyrosine kinase protein of the invention, incubating the protein with a ligand which is capable of binding to the protein and a suspected agonist or antagonist substance under conditions which permit the formation of ligand-receptor protein complexes, and assaying for ligand-receptor protein complexes, for free ligand or for non-complexed proteins.

The methods of the invention make it possible to screen a large number of potential ligands for their ability to bind to the novel receptor proteins of the present invention. The methods of the invention will also be useful for identifying potential stimulators or inhibitors of angiogenesis.

DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to the drawings in which:

Figure 1 shows the nucleotide and deduced amino acid sequence of the novel receptor tyrosine kinase protein of the invention;

Figure 2 shows the nucleotide and deduced amino acid sequence of a 1601 bp DNA segment of the invention;

Figure 3 shows a comparison of a portion of the deduced amino acid sequence of the novel receptor tyrosine kinase protein of the invention with that of other tyrosine kinases;

Figure 4 shows a Northern blot hybridization analysis of expression of the DNA segment of the invention in 12.5 day

murine embryonic heart;

Figure 5 shows the *in situ* hybridization analysis of expression of the DNA segment of the invention in the 12.5 day embryo;

Figure 6 shows the expression of the DNA segment of the invention precedes that of von Willebrand factor in 8.5 day embryos;

Figure 7 shows expression of the DNA segment of the invention in whole mount embryos(A., B., and C.); expression in Day 8.0 embryos (D.); mRNA distribution in a Day 9.5 embryo (E.); and *En2* expression in a Day 8 embryo (F.);

Figure 8 shows the expression of the DNA segment of the invention precedes that of von Willebrand factor in the developing leptomeninges and in particular the absence of immunohistochemical staining of von Willebrand factor in Day 12.5 leptomeninges (A); *in situ* detection of *tek* expression in Day 12.5 leptomeninges(B); staining of von Willebrand factor in Day 14.5 leptomeninges (C);

Figure 9 shows the expression of *tek* in adult vasculature and in particular bright field illumination of a section through the upper heart region of a 3 week-old mouse hybridized with an [^{35}S]-labelled *tek* probe (A); bright field illumination showing *tek* expression in endothelial cells lining the artery and vein respectively (B) and (C); and

Figure 10 shows the hierarchy of the endothelial cell lineage.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have isolated a novel protein

tyrosine kinase designated *tek*, expressed during murine cardiogenesis. By analyzing the segregation of an AccI restriction site polymorphism in AKR/J:DBA recombinant inbred mice, the present inventors mapped the *tek* locus to chromosome 4, between the *brown* and *pmv-23* loci. This region is syntenic with human chromosomal regions 1p22-23, 9q31-33, and 9p22-13. In mice and humans, these regions do not contain any previously described loci known to be involved with the biology of the endothelial cell lineage (Lyon, M.F. & Searle, A.G. *Genetic Variants and Strains of the Laboratory Mouse*, New York:Oxford University Press, 1989, 2nd, Ed.; O'Brien, 1990).

The novel gene products of the invention were identified as mouse receptor tyrosine kinase protein based on the structural homology of the protein to the known mouse and human tyrosine kinases. The deduced amino acid sequence of tek predicts that it encodes a putative receptor tyrosine kinase that contains a 21 amino acid kinase insert and which is most closely related in its catalytic domain to FGFR1 (mouse fibroblast growth factor) and the product of the ret proto-oncogene.

Northern blot hybridization analysis of RNA from 12.5 day embryonic heart using the 1.6 kb cDNA as probe suggested that the tek locus gives rise to at least 4 different transcripts of approximately 4.5, 2.7, 2.2, and 0.8 kb. Differential splicing of primary transcripts is known to occur for several genes encoding RTKs, including *met* (Rodrigues, G.A., Naujokas, M.A. & Park, M. (1991), *Mol.Cell.Biol.*, 11, 2962-2970), *trkB* (Middlemas, D.S., Lindberg, R.A. & Hunter, T. (1991), *Mol.Cell.Biol.*, 11, 143-153), *ret* (Tahira, T., Ishizaka, Y., Itoh, F., Sugimura, T. & Nagao, M. (1990), *Oncogene*, 5, 97-102), and *flg* (Reid *et al.*, 1990, Proc.Natl.Acad.Sci.,87,1596-1600; Bernard, O., Li, M. & Reid, H.H. (1991), *Proc.Natl.Acad Sci.USA*, 88, 7625-7629; Eisemann, A., Ahn, J.A., Graziani,

G., Tronick, S.R. & Ron, D. (1991), *Oncogene*, 6, 1195-1202; Fujita, H., Ohta, M., Kawasaki, T. & Itoh, N. (1991), *Biochem. Biophis. Res. Comm.*, 174, 946-951; Meng, B. & Reid, H.H. (1991), *Proc.Natl.Acad.Sci.*, 7625-7629), favoring the possibility that at least some of the smaller transcripts hybridizing with the *tek* cDNA are differentially spliced. The 4.5 kb *tek* transcript is of the appropriate size to encode a molecule with an extensive extracellular domain. In contrast, the smallest transcript, at 0.8 kb, is sufficient to encode only a significantly truncated version of the protein. Since this transcript was detected with a probe comprised entirely of sequences from the catalytic domain and 3' untranslated region, it is possible that the 0.8 kb message codes for an isoform completely lacking an extracellular domain. Truncated molecules of this type have recently been shown to be encoded by the *trkB* gene in rats (Middlemas *et al.*, 1991, Mol.Cell.Biol., 11, 143-153) and by *pdgfb* in murine ES cells (Vu, T.H., Martin, G.R., Lee, P., Mark, D., Wang, A. & Williams, L.T. (1989), *Mol.Cell.Biol.*, 9, 4563-4567). These small isoforms may act as catalytically deregulated molecules during periods of rapid growth (Middlemas *et al.*, 1991). The detection of multiple *tek* transcripts may indicate potential differential expression of different *tek* isoforms during embryogenesis.

In the adult and all stages of embryonic development examined, *tek* expression was restricted to cells of the endothelial lineage. Specifically, in situ hybridization analysis of adult tissues, as well as sectioned and whole mount embryos, showed that *tek* is specifically expressed in the endocardium, the leptomeninges and the endothelial lining of the vasculature from the earliest stages of their development. Moreover, examination of the morphology of *tek*-expressing cells, and staging of *tek* expression relative to that of the endothelial cell marker

von Willebrand factor, revealed that *tek* is express d prior to von Willebrand factor and appears to mark the embryonic progenitors of mature endothelial cells. *Thus, tek* encodes a novel putative receptor tyrosine kinase that may be critically involved in the determination and/or maintenance of cells of the endothelial lineage.

Overall, the pattern of expression observed in sectioned and whole mount mouse embryos was similar to that described previously for quail embryos stained with a monoclonal antibody specific for cells of the endothelial lineage (Pardanaud, L., Altmann, C., Kitos, P., Dieterlen-Lievre, F. & Buck, C.A. (1987). *Development*, 100, 339-349; Coffin, J.D. & Poole, T.J. (1988). *Development*, 102, 735-748). Thus, it is likely that orchestration of vascularization in the two vertebrate species is very similar. Studies on cell lineage relations carried out primarily in the chick (Noden, D.M. (1989), *Am.Rev.Respir.Dis.*, 140, 1097-1103, and Noden, D.M. (1990), *Ann. N.Y. Acad. Sci.*, 1, 236-249; O'Brien, S.J. *Genetic Maps, Locus Maps of Complex Genomes.* Cold Spring Harbor Laboratory Press, 1990) have established that endothelial cells are derived from angioblasts, which migrate from mesoderm and populate the embryo with precursor cells that eventually contribute to the formation of the intraembryonic blood vessels.

Figure 10 shows the hierarchy of the endothelial cell lineage. Horizontal bars denote the relationship between cellular determination and onset of expression of *tek* and von Willebrand factor within the lineage (adapted from (Wagner, R.C. (1980). *Adv.Microcirc.*, 9, 45-75). In the yolk sac, angioblasts are thought to originate from hemangioblasts, ill-defined cells of mesenchymal origin that are also believed to give rise to primitive blood cells in the developing blood islets. In the embryo, on the other hand, angioblasts are thought to arise directly

from cells of the mesenchymal anlage (Wagner, 1980).

The present inventors' work suggested that *tek* is expressed in the presumptive precursors of endothelial cells, the angioblasts. First, *tek* expression was detected in both von Willebrand factor-positive cells as well as cells that appear to be progenitors of endothelial cells. Second, *tek* expression was observed in cells of non-endothelial morphology that in the avian system have been identified previously as angioblasts. It may also be significant that in the 8.5 day embryo, *tek* expression was identified in cells extending beneath the ventral surface of somites (Figure 6J). Analysis of serial sections revealed that some of these *tek*-expressing cells were actually contiguous with the somites. These cells may correspond to those described by Beddington, R.S.P. & Martin, P. (1989), *Mol.Cell.Med.*, 6, 263-274 who showed in mouse tissue transplantation studies that *lacZ*-expressing somite tissue, while devoid of endothelial cells prior to transplantation, possess cells capable of migrating and contributing to the host vasculature. Taken together, the present inventors' work suggests that *tek* expression may constitute the earliest mammalian endothelial cell lineage marker described to date.

The restricted expression of *tek*, imposes constraints on the cellular range of activity of the putative Tek ligand, and suggests that the *tek* locus probably plays unique and important roles in the determination, migration, or proliferation of cells of the endothelial lineage.

As hereinbefore mentioned, the present inventors have identified and sequenced a cDNA sequence encoding a novel receptor tyrosine kinase protein designated tek. The DNA sequence and deduced amino acid sequence are shown in Figure 1. The DNA sequence and deduced amino acid sequence of a 1601 bp segment are shown in Figure 2.

DNA segments of the present invention encoding the novel receptor tyrosine kinase protein of the present invention or related or analogous sequences may be isolated and sequenced, for example, by synthesizing cDNAs from embryonic heart RNA by RT-PCR using degenerate oligonucleotide primers which amplify tyrosine kinase sequences such as the two degenerate tyrosine kinase oligonucleotide primers described by Wilks, A.F. ((1989) *Proc.Natl.Acad.Sci.*, 86, 1603-1607) and analyzing the sequences of the clones obtained following amplification. DNA segments of the present invention encoding the novel receptor tyrosine kinase protein of the present invention may also be constructed by chemical synthesis and enzymatic ligation reactions using procedures known in the art.

It will be appreciated that the invention includes nucleotide or amino acid sequences which have substantial sequence homology with the nucleotide and amino acid sequences shown in Figures 1 and 2. The term "sequences having substantial sequence homology" means those nucleotide and amino acid sequences which have slight or inconsequential sequence variations from the sequences disclosed in Figures 1 and 2 i.e. the homologous sequences function in substantially the same manner to produce substantially the same polypeptides as the actual sequences. The variations may be attributable to local mutations or structural modifications.

It will also be appreciated that a double stranded nucleotide sequence comprising a DNA segment of the invention or an oligonucleotide fragment thereof, hydrogen bonded to a complementary nucleotide base sequence, an RNA made by transcription of this doubled stranded nucleotide sequence, and an antisense strand of a DNA segment of the invention or an oligonucleotide fragment of the DNA segment, are contemplated within the scope of the

invention.

A number of unique restriction sequences for restriction enzymes are incorporated in the DNA segments identified in Figures 1, and these provide access to nucleotide sequences which code for polypeptides unique to the receptor tyrosine kinase protein of the invention. DNA sequences unique to the receptor tyrosine kinase protein of the invention or isoforms thereof, can also be constructed by chemical synthesis and enzymatic ligation reactions carried out by procedures known in the art.

The DNA segment of the present invention having a sequence which codes for the receptor tyrosine kinase protein of the invention, or an oligonucleotide fragment of the DNA segment may be incorporated in a known manner into a recombinant molecule which ensures good expression of the protein or part thereof. In general, a recombinant molecule of the invention contains the DNA segment or an oligonucleotide fragment thereof of the invention and an expression control sequence operatively linked to the DNA segment or oligonucleotide fragment. The DNA segment of the invention or an oligonucleotide fragment thereof, may be incorporated into a plasmid vector, for example, pECE.

The receptor tyrosine kinase protein or isoforms or parts thereof, may be obtained by expression in a suitable host cell using techniques known in the art. Suitable host cells include prokaryotic or eukaryotic organisms or cell lines, for example, yeast, E. coli and mouse NIH 3B cells may be used as host cells. The protein or parts thereof may be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

The DNA segments of the invention or oligonucleotide fragments of the DNA segments, allow those skilled in the art to construct nucleotide probes for use in the detection of nucleotide sequences in biological materials. A nucleotide probe may be labelled with a radioactive label which provides for an adequate signal and has sufficient half-life such as ^{32}P, ^{3}H, ^{14}C or the like. Other labels which may be used include antigens that are recognized by a specific labelled antibody, fluorescent compounds, enzymes, antibodies specific for a labelled antigen, and chemiluminescers. An appropriate label may be selected having regard to the rate of hybridization and binding of the probe to the nucleotide to be detected and the amount of nucleotide available for hybridization.

The nucleotide probes may be used to detect genes, preferably in human cells, that encode proteins related to or analogous to the receptor tyrosine kinase protein of the invention.

The receptor tyrosine kinase protein of the invention or parts thereof, may be used to prepare monoclonal or polyclonal antibodies. Conventional methods can be used to prepare the antibodies. As to the details relating to the preparation of monoclonal antibodies reference can be made to Goding, J.W., Monoclonal Antibodies: Principles and Practice, 2nd Ed., Academic Press, London, 1986. The polyclonal or monoclonal antibodies may be used to detect the receptor tyrosine kinase protein of the invention in various biological materials, for example they may be used in an Elisa, radioimmunoassay or histochemical tests. Thus, the antibodies may be used to quantify the amount of a receptor tyrosine kinase protein of the invention in a sample in order to determine its role in particular cellular events or pathological states.

The finding of a novel receptor tyrosine kinase which is

only expressed in cells of the endothelial lineage permits the identification of substances i.e. ligands, which may affect angiogenesis and/or maintenance of cells of the endothelial lineage. Therefore, in accordance with a method of the invention ligands, and natural and synthetic derivatives of such ligands, which are capable of binding to the receptor tyrosine kinase protein of the invention, isoforms thereof, or part of the protein may be identified. The method involves reacting the novel receptor kinase protein of the invention, isoforms thereof, or part of the protein with at least one ligand which potentially is capable of binding to the protein, isoform or part of the protein, under conditions which permit the formation of ligand-receptor protein complexes, and assaying for ligand-receptor protein complexes, for free ligand or for non-complexed proteins.

The ligand-receptor protein complexes, free ligand or non-complexed proteins receptor-ligand complex, may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the assay of the components, antibody against the receptor protein or the ligand, or a labelled receptor protein, or a labelled ligand may be utilized.

The receptor protein or ligand may be labelled with various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, biotin, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable

radioactive material include radioactive phosphorous ^{32}P, iodine I^{125}, I^{131} or tritium.

Radioactive labelled materials may be prepared by radiolabeling with ^{125}I by the chloramine-T method (Greenwood et al, Biochem. J. 89:114, 1963), the lactoperoxidase method (Marchalonis et al, Biochem. J. 124:921, 1971), the Bolton-Hunter method (Bolton and Hunter, Biochem. J. 133:529, 1973 and Bolton Review 18, Amersham International Limited, Buckinghamshire, England, 1977), the iodogen method (Fraker and Speck, Biochem. Biophys. Res. Commun. 80:849, 1978), the Iodo-beads method (Markwell Anal. Biochem. 125:427, 1982) or with tritium by reductive methylation (Tack et al., J. Biol. Chem. 255:8842, 1980).

Known coupling methods (for example Wilson and Nakane, in "Immunofluorescence and Related Staining Techniques", W. Knapp et al, eds, p. 215, Elsevier/North-Holland, Amsterdam & New York, 1978; P. Tijssen and E. Kurstak, Anal. Biochem. 136:451, 1984) may be used to prepare enzyme labelled materials. Fluorescent labelled materials may be prepared by reacting the material with umbelliferone, fluorescein, fluorescein isothiocyanate, dichlorotriazinylamine fluorescein, dansyl chloride, derivatives of rhodamine such as tetramethyl rhodamine isothiocyanate, or phycoerythrin.

The receptor protein or ligand used in the method of the invention may be insolubilized. For example, the receptor protein or ligand may be bound to a suitable carrier. Examples of suitable carriers are agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The

carrier may be in the shape of, for example, a tube, t st plate, beads, disc, sphere etc.

The insolubilized receptor protein or ligand may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

Conditions which permit the formation of ligand-receptor protein complexes may be selected having regard to factors such as the nature and amounts of the ligand and the receptor protein.

In a preferred embodiment of the method, ligands are identified which are capable of binding to and activating the novel receptor tyrosine kinase protein of the invention. In this method the ligands which bind to and activate the novel receptor tyrosine kinase receptor of the invention are identified by assaying for protein tyrosine kinase activity i.e by assaying for phosphorylation of the tyrosine residues of the receptor.

Protein tyrosine kinase activity may be assayed using known techniques such as those using antiphosphotyrosine antibodies and labelled phosphorous. For example, immunoblots of the complexes may be analyzed by autoradiography (^{32}P-labelled samples) or may be blocked and probed with antiphosphotyrosine antibodies as described in Koch, C.A. et al (1989) Mol. Cell. Biol. 9, 4131-4140.

As hereinbefore mentioned, the invention provides a method of using the novel proteins of the invention for assaying a medium for the presence of a substance that affects a tek effector system. In particular the method may be use to detect a suspected agonist or antagonist of a tek effector system. The agonist or antagonist may be an

endogenous physiological substance or it may be a natural or synthetic drug.

The term " tek effector system" used herein refers to the interactions of a ligand, and the receptor tyrosine kinase protein of the invention, and includes the binding of a ligand to the receptor protein or any modifications to the receptor associated therewith, to form a ligand/receptor complex and activating tyrosine kinase activity thereby affecting signalling pathways, particularly those involved in the regulation of angiogenesis.

In accordance with one embodiment, a method is provided which comprises providing a known concentration of a receptor tyrosine kinase protein of the invention, isoforms thereof, or part of the protein, incubating the protein, isoforms thereof, or part of the protein, with a ligand which is capable of binding to the protein, isoforms thereof, or part of the protein, and a suspected agonist or antagonist substance under conditions which permit the formation of ligand-receptor protein complexes, and assaying for ligand-receptor protein complexes, for free ligand or for non-complexed proteins.

The ligand-receptor complex, free ligand or non-complexed proteins may be assayed as described above. Suitable ligands used in the assay method may be identified using the methods described above. The ligand may be a natural ligand or a synthetic derivative having similar biological activity.

The invention also makes it possible to screen for antagonists that inhibit the effects of an agonist of a tek effector system, but do not have any biological activity in the tek effector system. Thus, the invention may be used to assay for a substance that competes for the same ligand-binding site on the novel receptor tyrosine

kinase protein of the invention.

It will be understood that the substances that can be assayed using the methods of the invention may act on one or more of the binding site on the receptor tyrosine kinase or the ligand, including agonist binding sites, competitive antagonist binding sites, non-competitive antagonist binding sites or allosteric sites.

The methods of the invention make it possible to screen a large number of potential ligands for their ability to bind to the novel receptor tyrosine kinase protein of the present invention. The methods of the invention are therefore useful for identifying potential stimulators or inhibitors of angiogenesis.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

The following materials and methods were utilized in the investigations outlined in the examples:

DNAs

AKR/J, DBA, and AKR/J x DBA recombinant inbred mouse DNAs were obtained from Jackson Labs (Bar Harbor, Maine), digested with *Acc*I, blotted to Zeta-Probe nylon membrane (Bio-Rad), and probed with the 1.6 kb *tek* cDNA labelled by random priming (Feinberg, A.P. & Vogelstein, B. (1983) *Analyt.Biochem.*, 132, 6-13). Hybridization was performed overnight at 65° in 200 mM sodium phosphate pH7.0, 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin (BSA), and 1 mM EDTA. Filters were washed twice at 55° in 2 x SSC (1 x SSC= 0.15 M NaCl, 0.015 M sodium citrate pH7.0) and 0.1% SDS and twice in 0.2 x SSC and 0.1% SDS, and exposed overnight to Kodak XAR-5 film.

Mice

Embryos and adult mouse tissues were obtained from random bred CD-1 stocks (Charles River, Quebec). Embryos were staged as Day 0.5 on the morning of a vaginal plug.

RNA purification and analysis

Total RNA was extracted from pools of 30 to 40 Day 9.5 and 12.5 murine embryonic hearts with RNAzol (CINNA/BIOTECX Lab. Int.), with some added modifications. Briefly, tissues were washed with ice cold phosphate buffered saline (PBS) and homogenized in 2.5 ml of RNAzol. Chloroform (250 µl) was added and the tubes were mixed vigorously and then chilled on ice for 15 min. The suspension was centrifuged for 15 min at 4° after which the aqueous phase was collected and re-extracted twice more with phenol/chloroform/isoamyl alcohol (25:24:1; vol:vol:vol). The RNA was precipitated with an equal volume of isopropanol, collected by centrifugation, and the pellet resuspended in diethylpyrocarbonate (DEPC)-treated 0.4 M sodium acetate, pH 5.2. The RNA were then reprecipitated with two volumes of 95% ethanol, washed with 70% and 95% ethanol, dried, and resuspended in DEPC treated 0.3 M sodium acetate, pH5.2. The RNA concentration was determined and the RNA stored at -70° until use.

Poly A - containing RNA was purified from a pool of 100 to 150 Day 12.5 murine embryonic hearts with a QuickPrep mRNA isolation kit (Pharmacia) as outlined by the supplier. For Northern blot hybridization, 5 µg of poly A - containing RNA from 12.5 day embryonic heart was electrophoresed through a formaldehyde-agarose gel and blotted to a Zeta-Probe nylon membrane (Bio-Rad) according to established protocols (Sambrook *et al.*, 1989, Molecular Cloning. Cold Spring Harbor Laboratory Press). The membrane was hybridized with a [^{32}P]-labelled antisense riboprobe synthesized from the 1.6 kb *tek* cDNA in run off

reactions with SP6 RNA polymerase (Promega).

Reverse Transcription Coupled to the Polymerase Chain Reaction (RT-PCR)

First strand cDNA was synthesized in a total reaction volume of 20 µl containing 20 µg of total RNA, 200 units of Mo-MLV-reverse transcriptase (BRL), either 1 µg of oligo-d$(T)_{18}$ (Day 12.5 RNA) (Boerhinger Mannheim) or 2 µg of random hexamer primers (Day 9.5 RNA) (Boerhinger Mannheim), 1 x PCR buffer (Cetus), 2.5 mM $MgCl_2$, 1 mM of dNTPs (Pharmacia), 40 units of RNAsin (Promega), and 12.5 mM dithiothreitol. The RNA was heated to 65°C for 10 min and cooled quickly on ice prior to addition to the reaction components. The reaction was allowed to proceed for 1 h at 37° and then terminated by heating for 5 min at 95°. For PCR, the reaction mixture was adjusted to a final volume of 100 µl containing 1 x PCR buffer, 1.5 mM $MgCl_2$, 800 µM dNTPs, and 1 µg of each of the two degenerate tyrosine kinase oligonucleotide primers described by Wilks, A.F. (1989) *Proc.Natl.Acad.Sci.*, 86, 1603-1607. Amplification was performed with a Ericomp thermocycler using the following parameters: denaturation for 2 min at 94°, annealing for 2 min at 42°, and extension for 4 min at 63°. After 40 cycles, the reaction products were collected by ethanol precipitation and electrophoresed through at 2% low-melt agarose (Sea Plaque) gel. In most cases a band of approximately 200 bp was visible within a background smear of ethidium bromide staining. This band was excised and recovered by three cycles of freeze-thaw in 100 µl of water. 10 µl of this solution was then subjected to a second round of PCR under the same conditions described above.

Cloning and sequencing of RT-PCR products.

After the second round of amplification, 10 µl of the reaction mixture were analyzed on a gel for successful

amplification. The remaining 90 µl were then ethanol precipitated, digested with *Eco*RI and *Bam*HI, gel purified, and ligated to pGEM7Zf+ (Promega) digested with the same enzymes. The ligation mixture was then transformed into MV1190 competent cells, individual amp^R colonies picked, plasmid DNA prepared, and the cDNA inserts analyzed by single track dideoxynucleotide sequencing (Sanger, F., Nicklen, S. & Coulson, A.R. (1977). *Proc.Natl.Acad.Sci.*, 74, 5463-5467). A single representative clone of each multiple isolate was sequenced in its entirety. Of the 58 clones analyzed, roughly 10% showed no sequence identity to tyrosine kinases and were disregarded.

Isolation of additional *tek* cDNA sequences.

Approximately 10^6 plaques from an amplified, random primed 13.5 day murine embryonic λgt10 cDNA library were hybridized with the 210 bp *tek* PCR product labelled with [^{32}P]-dCTP by PCR. Hybridization was carried out overnight at 55° in 50% formamide, 10% dextran sulfate (Pharmacia), 0.5 % BLOTTO, 4 x SSPE (1 x SSPE= 0.18 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH7.4), 100 µg/ml sheared salmon sperm DNA, and 2 x 10^6 cpm/ml of probe. Filters were washed at 55° twice in 2 x SSC containing 0.1% SDS and twice in 0.2 x SSC containing 0.1% SDS, dried, and exposed overnight to Kodak XAR-5 film. One clone was isolated from this screen and was found to contain a 1.6 kb cDNA. The sequence of the 1.6 kb cDNA was determined by the method of Sanger *et al.* (1977) from a set of anchored deletions generated with a standardized kit (Erase - A - Base, Promega).

In situ hybridization

Embryos isolated on Day 12.5 were dissected away from all extraembryonic tissues whereas embryos at earlier time points were recovered *in utero*. Embryos and adult tissues were fixed overnight in 4% paraformaldehyde, dehydrated with alcohols and xylenes, and embedded in paraffin. Tissues were sectioned at 6 µm thickness and mounted on 3-

aminopropyltriethoxysilane treated slides (Sigma). After removal of paraffin the samples were treated with predigested pronase (Boerhinger Mannheim), acetylated with triethanolamine, dehydrated, and hybridized according to the protocol described by Frohman, N.B., Boyle, M. & Martin, G.R. (1990), *Development*, 110, 589-607. Dark and bright field photomicroscopy was performed with a Leitz Vario Orthomat 2 photomicroscopic system. Adjacent sections probed with a *tek* sense probe produced no detectable signal above background.

Whole-mount *in situ* hybridizations were performed using a modification of existing procedures (Tautz, D. & Pfeifle, C. (1989). Chromosoma, 98,81-85;Hemmati-Brivanlou, A., Franck, D., Bolce, M.E., Brown, B.D., Sive, H.L. & Harland, R.M. (1990). *Development*, 110, 325-330; Conlon and Rossant, in prep.). The hybridization of single-stranded RNA probes labelled with digoxigenin was detected with antidigoxigenin antibodies coupled to alkaline phosphatase. The *En2* cDNA was prepared as set forth in Joyner A.L. & Martin, G.R. (1987). *Genes and Dev.*, 1, 29-38 and expression of *En2* is described in Davis, C.A., Holmyard, D.P., Millen, K.J. & Joyner, A.L. (1991) *Development*, 111:, 287-298.

Immunohistochemistry

Sections were stained immunohistochemically for von Willebrand factor with a commercially available kit (Biomeda). After color development, slides were counterstained with Harris hematoxylin.

EXAMPLE I

Isolation and characterization of *tek*

To identify and characterize tyrosine kinases expressed during murine cardiogenesis, cDNAs were synthesized from 9.5 and 12.5 day embryonic heart RNA by RT-PCR using

degenerate oligonucleotide primers previously demonstrated to amplify tyrosine kinase sequences preferentially (Wilks, A.F. 1989, *Proc.Natl.Acad.Sci.*, 86, 1603-1607). Considerable cellular differentiation and morphogenesis have occurred within the cardiac region of the embryo by Day 9.5. At this stage the heart has developed from the primordial mesoderm cells of the cardiac plate into a primitive bent tube structure, consisting of two endothelial tubes enclosed within the developing myocardium. Between Day 9.5 and 12.5 the heart undergoes additional complex morphological changes in association with the formation of the four chambers and septa characteristic of the adult heart. Sequence analysis of 58 clones obtained following amplification revealed that whereas roughly 10% did not contain sequence similarities to protein kinases the remainder corresponded to 5 distinct cDNAs (Table 1 - Identity and number of tyrosine kinase cDNA clones recovered from Day 9.5 and 12.5 murine embryonic heart by RT-PCR). Four of these cDNAs represented previously characterized tyrosine kinases including, *bmk*, *c-src*, *c-abl*, and the platelet derived growth factor receptor β-subunit *(pdgfrb)*. The isolation of *bmk*, *c-src*, and *c-abl* is consistent with the broad tissue distribution of these kinases (Wang, J.Y.J. & Baltimore, D. (1983). *Mol.Cell.Biol.*, 3, 773-779; Ben-Neriah *et al.*, *(1986)*. *Cell*, 44, 577-586; Holtzman, D., Cook, W. & Dunn, A. (1987). *Proc.Natl.Acad.Sci.*, 84, 8325-8329; Renshaw, M.W., Capozza, M.A. & Wang, J.Y.J. (1988). *Mol.Cell.Biol.*, 8, 4547-4551). The recovery from embryonic heart of *pdgfrb* at a relatively high frequency may indicate that *pdgfrb* plays an important role in cardiogenesis, as has been suggested by recent studies demonstrating that the addition of PDGF-BB to explants of axolotol cardiac field mesoderm stimulates the production of beating bodies (Muslin, A.J. & Williams, L.T. (1991). *Development*, 112, 1095-1101) the fifth cDNA, which was also isolated at high frequency, was novel and for reasons

that will become clear below was designated *tek*. The 210 bp RT-PCR-derived *tek* clone was subsequently used to isolate additional *tek* cDNA sequences.

Figure 1 shows the nucleotide and deduced amino acid sequence of tek. Figure 2 shows the nucleotide sequence of a 1.6 kb *tek* cDNA isolated from a 13.5 day mouse embryo cDNA library. Translation of this sequence reveals a single large open reading frame that terminates with TAG at nucleotide 907, followed by 696 nucleotides of 3' untranslated sequence. Several features of the deduced amino acid sequence suggest that the 1.6 kb *tek* cDNA encodes the cytoplasmic portion of a transmembrane RTK, consisting of the catalytic domain followed by a short carboxy-terminal tail of 33 amino acid residues.

Figure 3 shows a comparison of the deduced amino acid sequence of *tek* with that of other tyrosine kinases; Identical sequences are denoted by periods. Dashes were added to allow for optimal alignment. The kinase insert and conserved regions of the catalytic domain are indicated beneath the aligned sequences (Hanks, S.K., Quinn, A.M. & Hunter, T. (1988), *Science*, 241, 52). Comparative sequences shown are for human Ret (Takahashi, M. & Cooper, G.M. (1987). *Mol.Cell.Biol.*, 7, 1378-1385), and Jtk14 (Partanen, J., Mäkelä, T.P., Alitalo, R., Lehväslaiho, H. & Alitalo, K. (1990) Proc.Natl.Acad.Sci., 87, 8913-8917) and murine Flg (Reid, H.H., Wilks, A.F. & Bernard, O. (1990) *Proc.Natl.Acad.Sci.*, 87, 1596-1600).

As shown in Figure 3, the putative kinase domain contains several sequence motifs conserved among tyrosine kinases, including the tripeptide motif DFG, which is found in almost all known kinases, and the consensus ATP-binding site motifs GXGXXG followed by AXK 16 amino acid residues downstream (Hanks *et al.*, 1988). Transmembrane RTK's possess a methionine residue within the motif WMAIESL of

conserved region VIII of the catalytic domain (Hanks *et al.*, 1988) as does *tek*, and the catalytic domain is interrupted by a putative 21 amino acid kinase insert, a structural motif not found in cytoplasmic tyrosine kinases (Hanks *et al.*, 1988).

Comparison with other tyrosine kinases (Figure 3) reveals that the deduced *tek* amino acid sequence shows 42% sequence identity to the mouse fibroblast growth factor receptor Flg (Reid *et al.*, 1990; Safran, A., Avivi, A., Orr-Urtereger, A., Neufeld, G., Lonai, P., Givol, D. & Yarden, Y. (1990). *Oncogene*, 5, 635-643, Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). *Molecular Cloning*. Cold Spring Harbor Laboratory Press) and 45% to the transmembrane RTK encoded by the human *c-ret* proto-oncogene (Takahashi & Cooper, G.M. (1987). *Mol.Cell.Biol.*, 7, 1378-1385). In addition, striking sequence identity is observed to a 65 amino acid residue sequence encoded by Jtk14, a putative tyrosine kinase cDNA isolated from differentiating human K562 cells by RT-PCR (Partanen, J., Mäkelä, T.P., Alitalo, R., Lehväslaiho, H. & Alitalo, K. (1990) Proc.Natl.Acad.Sci., 87, 8913-8917). Taken together, the results suggest that *tek* encodes a novel RTK.

EXAMPLE II

Chromosomal mapping of the *tek* locus

Mapping of the *tek* locus was accomplished by monitoring the strain distribution pattern of an *Acc*I restriction site polymorphism in recombinant inbred (RI) mouse strains derived from matings between AKR/J (A) and DBA/2J (D) mice. The *tek* cDNA detects bands of 6.5, 6.1, 1.3 and 6.5, 3.1, 1.3 kb in DNA from the A and D strains, respectively. Southern blot hybridization analysis of DNA from 24 RI mice with the 1.6 kb cDNA probe, and comparison of the segregation pattern with the Jackson Laboratory data base, revealed 95.8% cosegregation between *tek* and

both *brown* and *pmv-23*, two loci that have previously been localized to mouse chromosome 4 (Lyon & Searle, 1989). Table 2 shows the cosegregation of the *tek*, *brown*, and *pmv-23* loci in A x D strains. In Table 2 for each RI strain, the symbol shown indicates the presence of an allele characteristic of the progenitor from which the strain was derived (A, AKR/J; D, DBA/2J). These data place *tek* between the *brown* and *pmv-23* loci within 3.8±1.9 centimorgans of each interval.

EXAMPLE III

Multiple *tek*-related transcripts are expressed in embryonic heart

tek expression in embryonic heart was examined by Northern blot hybridization using an antisense probe derived from the 1.6 kb *tek* cDNA. Figure 4 shows a Northern blot hybridization analysis of *tek* expression in 12.5 day murine embryonic heart; Arrows on the left denote the position of migration of 28 S and 18 S ribosomal RNAs obtained from adjacent lane loaded with total RNA. Figure 4 shows that the *tek* probe detects 4 transcripts of 4.5, 2.7, 2.2, and 0.8 kb in size in cardiac RNA from 12.5 day mouse embryos. These hybridizing species vary considerably in signal intensity, suggesting that they may differ in relative abundance, with expression of the 2.7 and 2.2 kb transcripts occurring at significantly higher levels than the 4.5 and 0.8 kb RNAs. While the exact relationship among these transcripts is unclear, it is possible that they arise by differential splicing, since the 1.6 kb *tek* cDNA detects a single genomic locus in mouse DNA by Southern blot hybridization at the same stringency.

EXAMPLE IV

In situ localization of *tek* expression during mouse embryogenesis

To determine which cell types express *tek* during

development, RNA *in situ* hybridization analyses were performed on mouse embryos with an antisense riboprobe synthesized from the 1.6 kb *tek* cDNA.

Figure 5 shows the *in situ* hybridization analysis of *tek* expression in the 12.5 day embryo; A. Dark field illumination of a para-sagittal section. Bar: 600µm. B. and C. Bright and dark field illumination respectively, of the heart region taken from a mid-sagittal section. Bar: 300 µm. IV and VI, fourth and sixth aortic arches; A, atrium; BA, basilar artery; CV, caudal vein; E, endocardium; L, liver; M, leptomeninges; Ma, mandible; My, myocardium; PC, pericardial cavity; RA, renal artery; SS, sino-auricular septum; SV, sinus venosus; V, ventricle.

Figure 5A shows that in 12.5 day mouse embryos, expression of *tek* is readily detected in the heart, the leptomeninges lining the brain and spinal cord, and the inner lining of major blood vessels, including the caudal vein and basilar and renal arteries. In addition, thin bands of hybridization are observed in the intersomite regions, corresponding to *tek* expression in the intersegmental vessels. Close examination of the region of the developing heart (Figure 5B and 5C) reveals that *tek* is expressed in the endocardium, as well as in cells lining the lumina of the atria, the IV and VI aortic arches, the sinus venosus, and the sino-auricular septum. In addition, *tek* expression is observed in numerous small blood vessels perforating the liver and mandible. These observations, together with the overall pattern of hybridization seen in the 12.5 day embryo, demonstrate that *tek* is expressed in the endothelial cells of the *tunica interna*, the innermost lining of the blood vessels; hence the designation *tunica interna* endothelial cell kinase, *tek*.

More detailed information on *tek* expression was obtained

through analysis of sections from earlier developmental stages. Hybridization to 6.5 and 7 day embryos revealed that while *tek* is expressed strongly in the inner lining of the small blood vessels and capillaries of the maternal decidua, no expression is observed in either the embryo itself or the ectoplacental cone. The absence of *tek* expression at these stages is consistent with the fact that at 6.5 to 7 days the embryo contains only a small amount of mesoderm from which endothelial cells are known to be derived.

Figure 6 shows the expression of *tek* precedes that of von Willebrand factor in 8.5 day embryos. Adjacent transverse sections through an 8.5 day embryo fixed *in utero* were either hybridized *in situ* with an [^{35}S]-labelled *tek* probe or stained immunohistochemically for von Willebrand factor. A. Bright field illumination of *tek* expression, Bar: 300 µm. B. Dark field illumination of section in A. C. High magnification of a blood island, slightly out of the field shown in A, depicting silver grains over flat, elongated cells of endothelial-like morphology, Bar: 50 µm. D. Adjacent section to A at higher magnification showing absence of expression of von Willebrand factor in the embryo, Bar: 100 µm. E. Adjacent section to A at higher magnification showing expression of von Willebrand factor in the endothelial lining of the blood vessels of the maternal decidua. Bar: 200 µm. F. High magnification of cephalic region in A showing silver grains over a large, round cell of angioblast-like morphology (arrow). Bar: 50 µm. G. Bright field illumination of a sagittal section of an 8.5 day embryo hybridized *in situ* with an [^{35}S]-labelled *tek* probe. Bar: 300 µm. H. Dark field illumination of G. I. Higher magnification of heart region in A showing silver grains over cells with endothelial- and angioblast-like morphology in the developing endocardium. Bar: 100 µm. J. Higher magnification of somite region in A showing *tek*-expressing

cells extending beneath, and possibly from, the ventral surface of the somites. Bar: 100 µm. A, amnion; Ag, presumptive angioblast; BI, blood island; D, maternal decidua; DA, dorsal aorta; E, endocardium; Ec, ectoplacental cone; En, endothelial cell; G, foregut; HV, head vein; NF, neural fold; S, somite; Y, yolk sac.

RNA *in situ* analysis of 8.0 day embryos revealed that *tek* expression first becomes detectable in the developing yolk sac and a few small clusters of cells in the cephalic mesenchyme. This expression becomes more pronounced by Day 8.5, at which time significant hybridization can be observed in the mesodermal component of the amnion (outer cell layer) and yolk sac (inner cell layer), as well as in the developing endocardium and the inner lining of the head veins and dorsal aortae (Figure 6A and 6B). In addition, sagittal sections reveal numerous focal areas of hybridization throughout the cephalic mesenchyme in regions thought to contain developing vasculature, as well as a small number of *tek*-expressing cells extending beneath the ventral surface of the somites (Figure 6H and 6J).

Whole mount *in situ* hybridization analysis confirmed and extended the above observations, as well as provided a three dimensional perspective on *tek* expression during embryogenesis. Figure 7 shows *tek* expression in whole mount embryos; A., B., C. and D. *tek* expression in Day 8.0 embryos. E. *tek* mRNA distribution in a Day 9.5 embryo. F. *En2* expression in a Day 8 embryo. I, II, III, first, second and third aortic arches; DA. dorsal aorta; E, endocardium; G, foregut pocket; H, heart; IS, intersegmental vessel; My, myocardium; ; NF, neural fold; OT; otic vesicle; V, vitelline vein; Y, yolk sac. Bars: 250 µm.

Consistent with the observations with sectioned material,

localized *tek* expression was not observed on embryonic Day 7. The first detectable expression was seen about the time of first somite formation when signal was observed in the yolk sac, head mesenchyme, and heart. In Day 8.5 embryos, *tek* was found to be expressed in these same areas, and in the paired dorsal aortae, the vitelline veins, and in the forming intersegmental vessels (Figure 7). By this time, *tek* expression was clearly confined to blood vessels within the embryo. On Day 9, *tek* expression was seen in addition, in the aortic arches and expression was very striking in the endocardium (Figure 7E). Control hybridizations with an *En-2* probe demonstrated the specificity of *tek* RNA detection (Figure 7F).

EXAMPLE V

Expression of *tek* in endothelial cell progenitors

The observation that *tek* is expressed between Day 8.0 and 8.5 in focal regions thought to represent developing blood vessels raised the possibility that *tek* might be expressed in endothelial cell progenitors. Indeed, close inspection of hybridized sections from 8 to 8.5 day embryos revealed that while the expression of *tek* in the maternal decidua is restricted to cells of an endothelial cell morphology, *tek* expressing cells in the embryo are of two morphologically distinct cell types. In the developing blood islands of the yolk sac, where *tek* expression is first detected, silver grains are localized predominantly to elongated cells with characteristic endothelial cell morphology (Figure 6C). In contrast, within the cephalic mesenchyme, silver grains are frequently observed over large, round cells that, on the basis of similar morphology to cells described during avian embryogenesis (Pardanaud *et al.*, 1987; Coffin & Poole, 1988; Noden, 1989; Noden, 1991), correspond to angioblasts, the presumptive progenitor of endothelial cells (Figure 6F).

Both cell types are observed in the developing endocardium (Figure 6I) which, at later stages, is known to contain only fully mature endothelial cells.

To characterize more precisely the staging of *tek* expression within the endothelial lineage, sections adjacent to those used for *in situ* hybridization were stained immunohistochemically for von Willebrand factor, a well characterized marker of mature endothelial cells (Jaffe, E.A., Hoyer, L.W. & Nachman, R.L. (1973). *J.Clin.Invest.*, 52, 2757-2764; Hormia, M., Lehto, V.-P. & Virtanen, I. (1984), *Eur.J.Cell.Biol.*, 33, 217-228). Figure 6B and H shows that whereas *tek* is expressed in both the maternal decidua and the embryo at Day 8.5, expression of von Willebrand factor is observed only in the *tek*-expressing, vascular endothelial cells of the maternal decidua (Figure 6D and 6E). Hence *tek* expression precedes that of von Willebrand factor during embryogenesis. The same scenario is observed at later developmental stages during vascularization of individual organs.

Figure 8 shows the expression of *tek* precedes that of von Willebrand factor in the developing leptomeninges; A. Absence of immunohistochemical staining of von Willebrand factor in Day 12.5 leptomeninges. Arrow denotes a large blood vessel faintly positive for von Willebrand factor. B. *In situ* detection of *tek* expression in Day 12.5 leptomeninges. C. Staining of von Willebrand factor in Day 14.5 leptomeninges. Day 14.5 leptomeninges were positive for *tek* expression (not shown). M, leptomeninges. Bars: 200 µm.

Figure 8 shows that in the 12.5 day embryo, the developing leptomeninges hybridizes strongly with *tek* but fails to stain positive for von Willebrand factor. By Day 14.5, however, expression of von Willebrand factor can be

readily detected in the leptomeninges. Assuming that there is not a significant lag between transcription and translation of von Willebrand factor, these observations, together with those on the morphology of *tek*-expressing cells, suggest that *tek* is expressed in both mature endothelial cells and their progenitors.

EXAMPLE VI

tek is expressed in adult vasculature

While the above results establish that *tek* is expressed during vascularization of the embryo, it was also of interest to determine whether expression of *tek* is maintained in endothelial cells of the adult. *In situ* hybridization analysis of a section through the heart region of a 3 week-old mouse revealed that *tek* is expressed in the endocardium as well as in the endothelial lining of major blood vessels, both arteries and veins, connecting with the adult heart (Figure 9).

Figure 9 shows the expression of *tek* in adult vasculature. A. Bright field illumination of a section through the upper heart region of a 3 week-old mouse hybridized with an [^{35}S]-labelled *tek* probe. Bar: 20 µm. B. and C. Bright field illumination showing *tek* expression in endothelial cells lining the artery and vein respectively. Bar: 1 µm. Immunohistochemical staining of adjacent sections revealed that structures positive for *tek* expression also stained positive for von Willebrand factor. A, artery; Bl, extravasated blood; T, trachea; V, vein.).

The intensity of the hybridization signal observed for these structures is considerably lower than that observed for the endocardium and blood vessels of 12.5 day embryos hybridized and processed in parallel. This could indicate that mature endothelial cells, which are thought to be

resting, have a different quantitative or qualitative requirement for expression of *tek*.

Table 1: Protein tyrosine kinase cDNAs isolated by RT-PCR

Embryonic Age (Days)	cDNA				
	tek	*pdgfrb*	*c-abl*	*c-src*	*bmk*
9.5	26	7	2	1	1
12.5	5	10	-	-	-

TABLE 2. Cosegregation of the *tek*, *brown*, and *pmv-23* loci in A x D strains.

	A x D strain																								
Locus	1	2	3	6	7	8	9	10	11	12	13	14	15	16	18	20	21	22	23	24	25	26	27	28
tek	D	D	A	D	D	A	A	A	D	A	D	A	D	D	D	D	A	D	D	A	D	D	D	D
brown	D	D	A	D	D	A	A	A	D	A	A	A	D	D	D	D	A	D	A	A	D	D	D	D
pmv-23	D	D	A	D	D	A	D	A	D	A	D	D	D	A	D	D	A	D	D	A	D	D	D	A

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A purified and isolated DNA segment having a sequence which codes for a receptor tyrosine kinase protein which is expressed only in cells of endothelial lineage, or an oligonucleotide fragment of the DNA segment which is unique to the receptor tyrosine kinase protein.

2. A purified and isolated DNA segment having a sequence which codes for a receptor protein having an amino acid sequence which has substantial homology with the amino acid sequence as shown in Figure 1.

3. A purified and isolated DNA segment having a sequence having substantial sequence homology with the nucleotide sequence as shown in Figure 1.

4. A purified and isolated double stranded nucleotide sequence comprising a DNA segment as claimed in claim 1, hydrogen bonded to a complementary nucleotide base sequence.

5. A recombinant molecule adapted for transformation of a host cell comprising a DNA segment as claimed in claim 1 and an expression control sequence operatively linked to the DNA molecule.

6. A transformant host cell including a recombinant molecule comprising a DNA segment as claimed in claim 1 and an expression control sequence operatively linked to the DNA segment.

7. A method for preparing a receptor tyrosine kinase protein utilizing a purified and isolated DNA segment as claimed in claim 1.

8. A substantially pure receptor tyrosine kinase protein which is expressed only in cells of the endothelial lineage or isoforms thereof.

9. A substantially pure protein having an amino acid sequence which has substantial homology with the amino acid sequence as shown in Figure 1.

10. A protein encoded by the purified and isolated DNA segment as claimed in claim 1 or 2.

11. A method for identifying ligands which are capable of binding to a receptor tyrosine kinase protein which is only expressed in cells of the endothelial lineage, isoforms thereof, or part of the protein, comprising reacting a receptor kinase protein which is only expressed in cells of the endothelial lineage, isoforms thereof, or part of the protein, with at least one ligand which potentially is capable of binding to the protein, isoform or part of the protein, under conditions which permit the formation of ligand-receptor protein complexes, and assaying for ligand-receptor protein complexes, for free ligand or for non-complexed proteins.

12. The method as claimed in claim 12, wherein ligands are identified which are capable of binding to and activating the a receptor tyrosine kinase protein which is expressed only in cells of the endothelial lineage, isoforms thereof, or part of the protein.

13. The method as claimed in claim 12, wherein the ligands which bind to and activate the novel receptor tyrosine kinase receptor of the invention are identified by assaying for protein tyrosine kinase activity.

14. A method of assaying a medium for the presence of a substance that affects the interaction of a receptor

tyrosine kinase protein which is expressed only in cells of the endothelial lineage.

15. A method for assaying a medium for the presence of an agonist or antagonist of the interaction of a receptor tyrosine kinase protein which is expressed only in cells of the endothelial lineage, which comprises providing a known concentration of a receptor tyrosine kinase protein which is only expressed in cells of the endothelial lineage, incubating the protein with a ligand which is capable of binding to the protein and a suspected agonist or antagonist substance under conditions which permit the formation of ligand-receptor protein complexes, and assaying for ligand-receptor protein complexes, for free ligand or for non-complexed proteins.

16. A method as claim in claim 11 or 15, wherein the receptor tyrosine kinase protein has an amino acid sequence which has substantial homology with the amino acid sequence as shown in Figure 1.

17. A method as claim in claim 11 or 15, wherein the receptor tyrosine kinase protein is encoded by the purified and isolated DNA segment as claimed in claim 1.

18. A method as claim in claim 11 or 15, wherein the receptor tyrosine kinase protein is encoded by a DNA segment which has substantial sequence homology with the nucleotide sequence as shown in Figure 1.

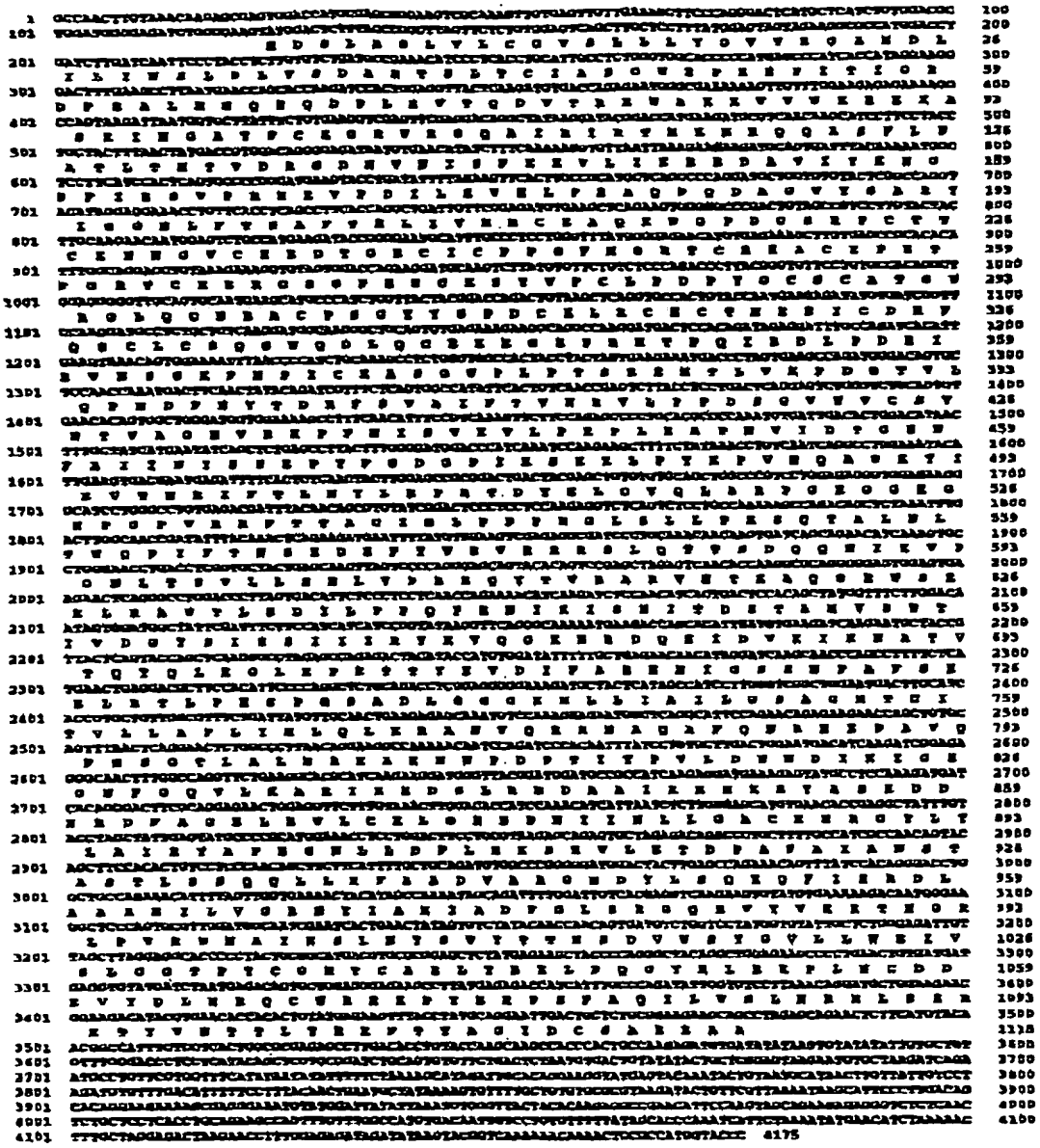

FIGURE 1

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   1  ATCAAGTTTCAAGACGTGATCGGAGAGGGCAACTTTGGCCAGGTTCTGAAGGCACGCATCAAGAAGGATG   70
      I  K  F  Q  D  V  I  G  E  G  N  F  G  Q  V  L  K  A  R  I  K  K  D  G
  71  GGTTACGGATGGATGCCGCCATCAAGAGGATGAAAGAGTATGCCTCCAAAGATGATCACAGGGACTTCGC  140
        L  R  M  D  A  A  I  K  R  M  K  E  Y  A  S  K  D  D  H  R  D  F  A
 141  AGGAGAACTGGAGGTTCTTTGTAAACTTGGACACCATCCAAACATCATTAATCTCTTGGGAGCATGTGAA  210
        G  E  L  E  V  L  C  K  L  G  H  H  P  N  I  I  N  L  L  G  A  C  E
 211  CACCGAGGCTATTTGTACCTAGCTATTGAGTATGCCCCGCATGGAAACCTCCTGGACTTCCTGCGTAAGA  280
        H  R  G  Y  L  Y  L  A  I  E  Y  A  P  H  G  N  L  L  D  F  L  R  K  S
 281  GCAGAGTGCTAGAGACAGACCCTGCTTTTGCCATCGCCAACAGTACAGCTTCCACACTGTCCTCCCAACA  350
        R  V  L  E  T  D  P  A  F  A  I  A  N  S  T  A  S  T  L  S  S  Q  Q
 351  GCTTCTTCATTTTGCTGCAGATGTGGCCCCGGGGGATGGACTACTTGAGCCAGAAACAGTTTATCCACAGG  420
        L  L  H  F  A  A  D  V  A  R  G  M  D  Y  L  S  Q  K  Q  F  I  H  R
 421  GACCTGGCTGCCAGAAACATTTTAGTTGGTGAAAACTACATAGCCAAAATAGCAGATTTTGGATTGTCAC  490
        D  L  A  A  R  N  I  L  V  G  E  N  Y  I  A  K  I  A  D  F  G  L  S  R
 491  GAGGTCAAGAAGTGTATGTGAAAAAGACAATGGGAAGGCTCCCAGTGCGTTGGATGGCAATCGAATCACT  560
        G  Q  E  V  Y  V  K  K  T  M  G  R  L  P  V  R  W  M  A  I  E  S  L
 561  GAACTATAGTGTCTATACAACCAACAGTGATGTCTGGTCCTATGGTGTATTGCTCTGGGAGATTGTTAGC  630
        N  Y  S  V  Y  T  T  N  S  D  V  W  S  Y  G  V  L  L  W  E  I  V  S
 631  TTAGGAGGCACCCCCTACTGCGGCATGACGTGCGCCGGAGCTCTATGAGAAGCTACCCCAGGGCTACAGGC  700
        L  G  G  T  P  Y  C  G  M  T  C  A  E  L  Y  E  K  L  P  Q  G  Y  R  L
 701  TGGAGAAGCCCCTGAACTGTGATGATGAGGTGTATGATCTAATGAGACAGTGCTGGAGGGAGAAGCCTTA  770
        E  K  P  L  N  C  D  D  E  V  Y  D  L  M  R  Q  C  W  R  E  K  P  Y
 771  TGAGAGACCATCATTTGCCCAGATATTGGTGTCCTTAAACAGGATGCTGGAAGAACGCAAGACATACGTG  840
        E  R  P  S  F  A  Q  I  L  V  S  L  N  R  M  L  E  E  R  K  T  Y  V
 841  AACACCACACTGTATGAGAAGTTTACCTATGCAGGAATTGACTGCTCTGCGGAAGAAGCAGCCTAGAGCA  910
        N  T  T  L  Y  E  K  F  T  Y  A  G  I  D  C  S  A  E  E  A  A  *
 911  GAACTCTTCATGTACAACGGCCATTTCTCCTCACTGGCGCGAGAGCCTTGACACCTGTACCAAGCAAGCC  980
 981  ACCCACTGCCAAGAGATGTGATATATAAGTGTATATATTTGTGCTGTGTTTGGGACCCTCCTCATACAGCT 1050
1051  CGTGCGGATCTGCAGTGTGTTCTGACTCTAATGTGACTGTATATACTGCTCGGAGTAAGAATGTGCTAAG 1120
1121  ATCAGAATGCCTGTTCGTGGTTTCATATAATATATTTTTCTAAAAGCATAGATTGCACAGGAAGGTATGA 1190
1191  GTACAAATACTGTAATGCATAACTTGTTATTGTCCTAGATGTGTTTGACATTTTTCCTTTACAACTGAAT 1260
1261  GCTATAAAAGTGTTTTGCTGTGTGCGCGTAAGATACTGTTCGTTAAAATAAGCATTCCCTTGACAGCACA 1330
1331  GGAAGAAAAGCGAGGGAAATGTATGGATTATATTAAATGTGGGTTACTACACAAGAGGCCGAACATTCCA 1400
1401  AGTAGCAGAAGAGAGGGTCTCTCAACTCTGCTCCTCACCTGCAGAAGCCAGTTTGTTTGGCCATGTGACA 1470
1471  ATTGTCCTGTGTTTTTATAGCACCCAAATCATTCTAAAATATGAACATCTAAAAACTTTGCTAGGAGACT 1540
1541  AAGAACCTTTGGAGAGATAGATATAAGTACGGTCAAAAAACAAAACTGCGCCATGGTACCC 1601
```

FIGURE 2

05/18/99 *** NOT ENTERED *** 11:43 FAX 416 862 7661

```
Tek     IKFQDVIGEGNFGQVLKAR---IKKD  GLR--MDAAIKMMKEYASKD  DHRDFAGELEVLCKLG  MHPNIINLLGACEHR  GYLYLAIEYAPHG        85
Jtk14
Ret     LVLGKTL...E..K.V..TAFHL.GR  AGY--TTV.V.ML..N..PS  EL..LLS.FK...QV  N..HV.K.Y...SQD  .P.L.IK...KY.       543
FlgM    LVLGKPL...C....VL.EAIGLD..  KPNRVTKV.V.ML.SD.TEK  .LS.LIS.M.MMKMI.  K.K.........TQD  .P..VIV...SK.       567
                      I                        II                III              IV              V
Tek     NLLDFL  RKSRVLETDPAFAIANSTAST--------  LSSQQLLHFAADVARGMDYLSQKQFIHRDLAARNILVGENY  IAKIADFGLSR---GQEV    169
Jtk14                                                                                .....V.....L  AS.....---.E..     32
Ret     S.RG..  .E..KVGPGYLGSGGSRNS.SLDHPDERA  .TMGD.IS..WQISQ..Q..AEMKLV..........A.GR  KM..S.....DVYEEDP    638
FlgM    ..REV.  QAR.PPGLEYCYNPSHNPEEQ--------  ...KD.VSC.YQ.....E..AS.KC.........V..T.DN  VM.......A.DIHHIDY    654
                                Insert                                  VI                              VII
Tek     YVKK  TMGRLPVRWMAIESL  NYSVYTT-NSDVWSYGVLLWEIVSLGGTPYCG  NTCADVYEKLPQGYRLEKPLN  CDDEVYHVMRQCWREKPYER    260
Jtk14   ....  ..............  .......-K....F.                                                                    65
Ret     ...R  SQ..I..K.......  FDMI...-Q.....F.......T...N..P.  IPPERLFNL.KT.H.M.R.D.  .SE.N.R..L...KQE.DK.    729
FlgM    .K.T  .N.....K...P.A.  FDRI..-HQ.....F.......FT...S..P.  VPVEELFKL.KE.H.MD..S.  .TM.L.MM..Q..HAV.SQ.    745
                        VIII                            IX                                  X                   XI
Tek     PSFAQLLVSL  -NRNL-EERKTYVNTTLYEKFTYAGI-DC-SAEEAA  301
Jtk14
Ret     .V..D.SKD.  E.M.VK--.RD.LDLAASTPSDSLIYD.GL.E..TP  772
FlgM    .T.K.LVED.  DHIV..TSNQE.LDLSIPLDQYSPSFP.TR.STCSS  790
```

FIGURE 3

2085291

045

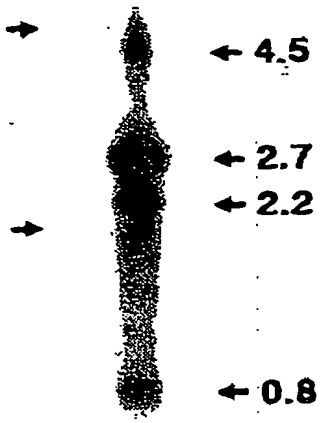

FIGURE 4

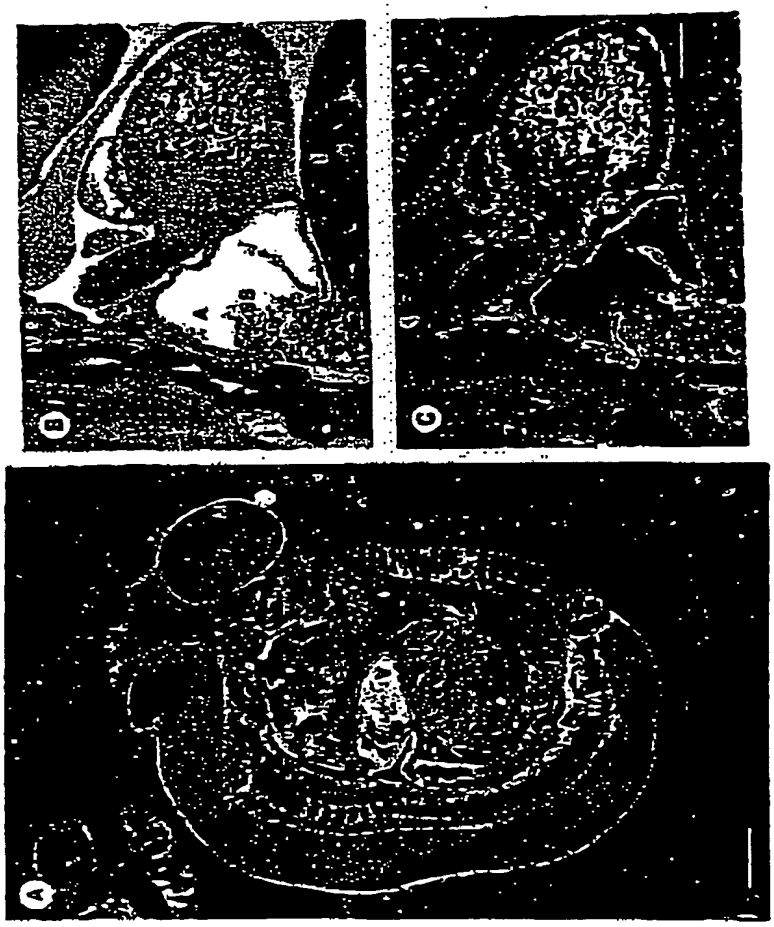

FIGURE 5

085291

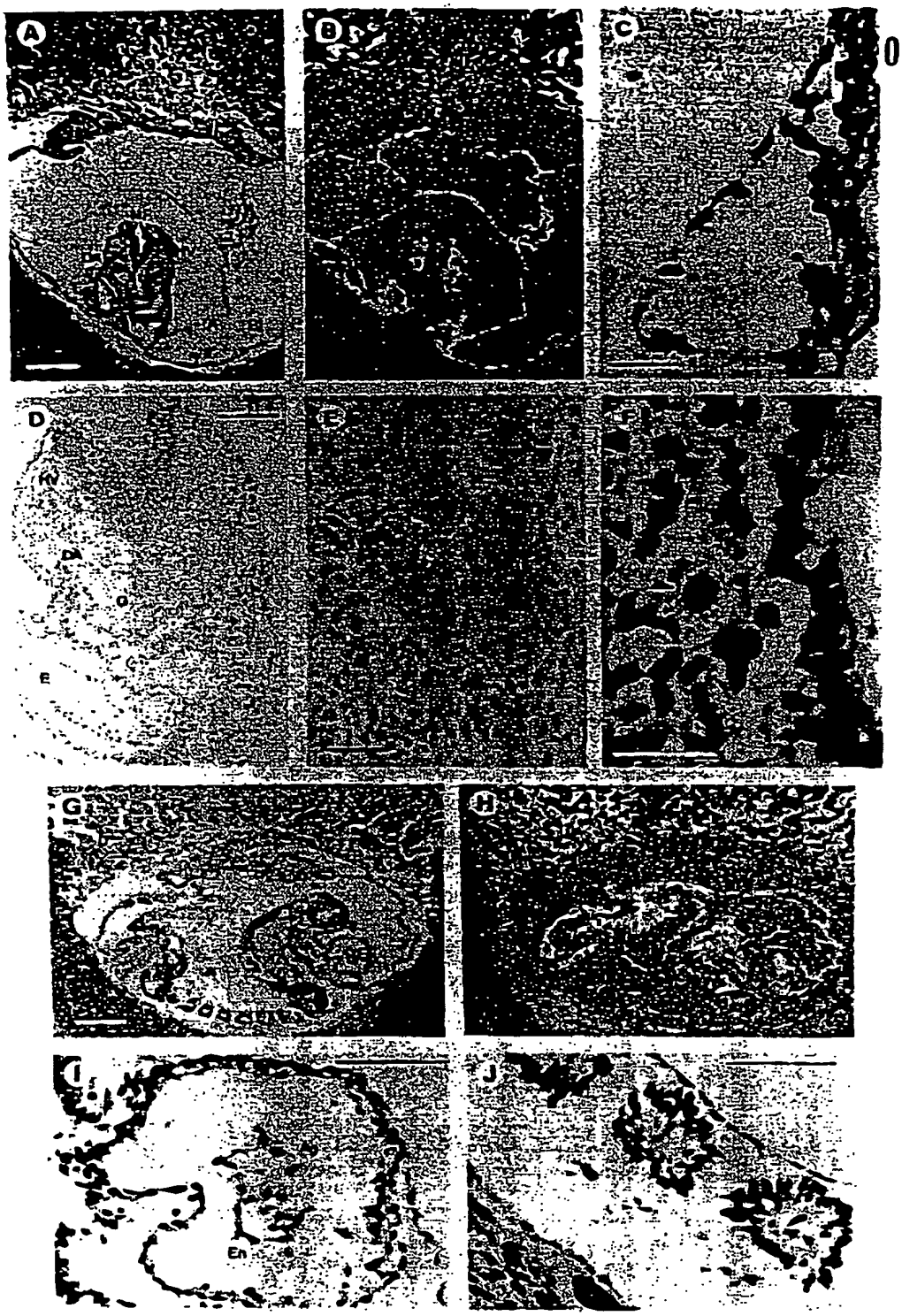

FIGURE 6

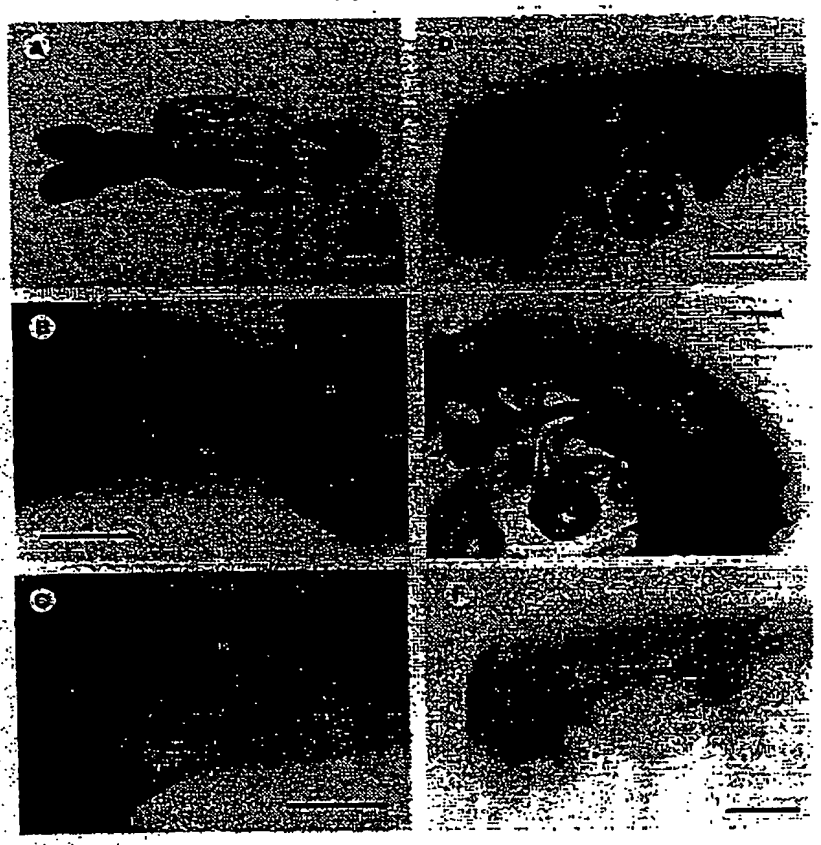

FIGURE 7

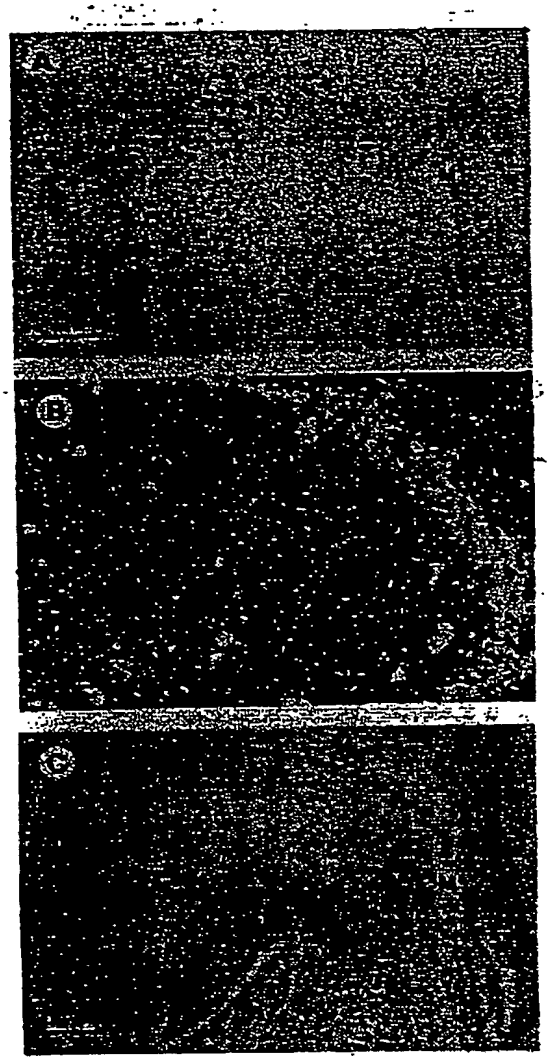

FIGURE 8

FIGURE 9

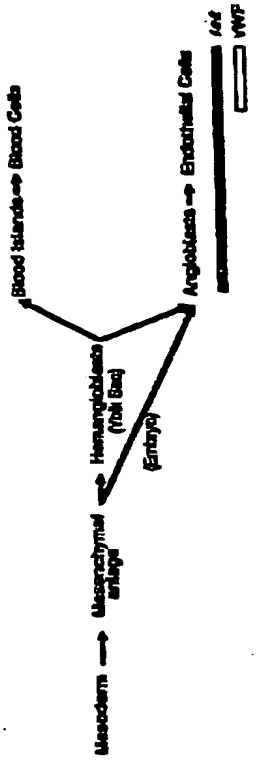
Blood Islands → Blood Cells
Angioblasts → Endothelial Cells
Flk1
VWF
Hemangioblasts (Yolk Sac) (Embryo)
Mesenchymal anlagen
Mesoderm

FIGURE 10